Original Contribution

DIFFERENTIAL INDUCTION AND DECAY OF MANGANESE SUPEROXIDE DISMUTASE mRNAs

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Abstract—Human manganese superoxide dismutase (MnSOD) is encoded by two mRNAs of 4 and 1 kb, respectively. These mRNAs are transcribed from the same gene and have an identical coding sequence, but differ in the 3' untranslated sequence because of alternate polyadenylation. Tumor necrosis factor-α (TNF) induced both 4- and 1-kb mRNAs in all the human cell lines examined. However, the relative expression of these mRNAs varied significantly among different cell lines after an 8-h treatment with TNF. Therefore, the time course of induction by TNF and the decay of MnSOD mRNAs after TNF removal were analyzed. The rate of accumulation of the 4-kb mRNA was initially much greater than that of the 1-kb mRNA, suggesting that the 4-kb mRNA was produced faster than the 1-kb mRNA. The rapid accumulation of the 4-kb mRNA was offset after a few hours by an enhanced rate of decay. The half-life of the 4-kb mRNA was ~2–4 h in different cells while that of the 1-kb mRNA was ~10–12 h. This different half-life of mRNAs that encode the same protein suggests that their relative expression is also regulated by a post-transcriptional mechanism affecting their turnover. Additional evidence supporting the differential decay of the two MnSOD mRNAs was obtained by incubation in a rabbit reticulocyte cell-free system; the 4-kb mRNA decayed rapidly while the 1-kb mRNA appeared to be stable.

Keywords—Superoxide dismutase, Tumor necrosis factor, mRNA stability, Free radicals

INTRODUCTION

The toxicity of oxygen has been shown to be directly related to the production of oxygen-dependent free radicals. These radical species target sulfhydryl-containing proteins, nucleic acids, and lipids; the accumulation of toxic byproducts has been implicated in a variety of pathological states including autoimmune disorders, carcinogenesis, and aging.[1] Cells are protected from free radical damage by a variety of antioxidant enzymes and chemicals. The superoxide dismutases (SOD) are key components of this antioxidant defense system and are found in both prokaryotic and eukaryotic cells.[2] Manganese SOD (MnSOD) is one of three mammalian metalloproteins that catalyze the dismutation of superoxide radicals to hydrogen peroxide and oxygen. MnSOD is localized in the mitochondrial matrix,[3] presumably to remove superoxide produced during normal mitochondrial function. Changes in the content of SOD in mammalian cells have been observed under a variety of conditions and appear to be highly regulated (e.g., during development and differentiation,[4,5] under hypoxic and hyperoxic conditions,[6] after X-irradiation[7] and treatment with paraquat,[8] 2,4-dinitrophenol, phorbol esters,[9] lipopolysaccharide,[10] or cytokines).[11-13] Tumor necrosis factor (TNF), a cytokine that is cytotoxic for some tumor cells, specifically induces high MnSOD levels in mammalian cells both in vivo and vitro.[12] The mechanism of TNF cytotoxicity for tumor cells is unclear, but it may involve the production of reactive oxygen species.[14,15] Therefore, the induction of MnSOD may protect cells from the cytotoxic activity of TNF.

The regulation of MnSOD biosynthesis is complicated by the existence of two distinct MnSOD mRNA species of 4 and 1 kb, respectively.[16] These MnSOD mRNAs have an identical coding region but differ in the length of their 3' untranslated region (3'UTR), because of alternate polyadenylation.[17] A unique feature of the 4-kb transcript is the presence of an AU-rich sequence, which has been associated with mRNA instability.[18,19] Variability in the relative level of the two MnSOD mRNAs has been previously reported,[5,9,20] but the reason for this variability is unknown.

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In the present study, we compared the expression of MnSOD mRNAs among human cell lines in response to TNF. The expression of the 4- and 1-kb mRNAs varied among these cells, and the 4-kb mRNA had a shorter half-life than the 1-kb mRNA both in intact cells and in a cell-free system. We propose that the functional significance of the different 3'UTR sequences is related to differential mRNA stability; this may play a major role in regulating the expression of MnSOD.

MATERIALS AND METHODS

Cell culture and treatment

Human glioblastoma A172, fibrosarcoma HT-1080, and osteosarcoma HOS cells were grown in DMEM supplemented with 10% heat-inactivated horse serum; melanoma SK-MEL-109 cells were grown in F-12 and Eagle's medium (1:1) supplemented with 8% fetal calf serum. All cells were routinely grown in 25 cm² flasks containing 5 ml of medium. Human diploid fibroblasts RIG cells were derived from newborn foreskin and were cultured in M199 medium supplemented with 10% fetal bovine serum. All tissue culture media and sera were purchased from Gibco Life Technologies (Baltimore, MD). The cells were treated with human recombinant TNF which was a gift of Dr. T. Nishihara of the Suntory Institute for Biomedical Research, Osaka, Japan.

RNA extraction and analysis

Approximately $1–5 \times 10^6$ cells were lifted from a 25 cm² culture flask by adding 1 mM ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline, pH 7.2. The cells were collected by centrifugation and lysed with 0.1 ml of 1% NP-40 in 10 mM Tris-HCl buffer, pH 7.4, by briefly vortexing. Nuclei were removed by centrifugation at $10,000 \times g$. To the supernatant were added 0.3 M sodium acetate, 1% SDS, 20 mM Tris-HCl buffer, pH 7.4, 10 mM EDTA, and 150 μl of phenol:chloroform:isoamyl alcohol. The suspension was vortexed for 10 s and centrifuged. The aqueous layer was again extracted with phenol and then with chloroform:isoamyl alcohol. The RNA was precipitated at $-20°$C with ethanol, recovered by centrifugation, washed with 70% ethanol, dried, resuspended in 100% deionized formamide, and stored at $-20°$C. The RNA was analyzed as previously described.[21]

cDNA probes

A pGEM-4 plasmid containing 750 base-pair of cDNA for human MnSOD was a gift of Dr. David Goeddel, Genentech. This plasmid was digested with XbaI and PstI. The digest was fractionated by electrophoresis in low-melting-point agarose, and the band corresponding to the cDNA insert was excised, melted, and labeled with 20 μCi of $[\alpha-^{32}P]$dCTP using the Klenow fragment of DNA polymerase according to the random primer method.[22] The cDNA probes for human β-actin and glyceraldehyde-phosphate dehydrogenase (GAPDH) were labeled in the same way.

Reticulocyte lysate experiments

Total cellular RNA was prepared from HOS cells treated for 4–6 h with TNF by the guanidinium hydrochloride method.[23] Poly(A⁺) RNA was isolated by hybridization to oligo(dT)-cellulose (Type 3; Collaborative Research) as described.[24] The poly(A⁺) RNA was incubated at 30°C with 30 μl of unsupplemented reticulocyte lysate prepared in our laboratory.[25] The incubation was stopped by the addition of 170 μl of 0.5% SDS, 100 mM NaCl, 50 mM Tris-HCl buffer, pH 7.4, and 10 mM EDTA. The RNA was extracted and ethanol-precipitated as described above.

Superoxide dismutase activity gel analysis

Cells were harvested from one 25 cm² tissue culture flask with 1 ml of phosphate-buffered saline, pH 7.2, containing 1 mM EDTA. After a brief centrifugation, the cell pellet was resuspended in 50 μl of phosphate buffer, pH 7.8, and sonicated for 30 s. The lysate was centrifuged at $10,000 \times g$ for 10 min and the supernatant collected; this centrifugation was repeated twice. The protein concentration of the final supernatant was determined using the BCA protein reagent (Pierce, Rockford, IL). SOD activity was assayed as previously described.[26] Briefly, 25 μg of protein from cell lysate were fractionated by electrophoresis in a discontinuous polyacrylamide gel, consisting of a 3% stacking gel (pH 6.8) and a 10% running gel (pH 8.8). To visualize SOD activity, the gels were incubated for 15 min in the dark with 2.5 mM nitroblue tetrazolium, 30 mM TEMED, 28 μM riboflavin, 50 mM phosphate buffer, pH 7.8, washed twice in deionized water, and then exposed to fluorescent light until clear zones of SOD activity were observed.

RESULTS

Kinetics of synthesis and turnover of MnSOD mRNAs

Initial studies showed differences in the relative proportion of 4-kb to 1-kb MnSOD mRNA induced by TNF in various cell lines (Fig. 1) (e.g., the two mRNAs were present in approximately equal amounts in HOS and SK-MEL-109 cells, while the
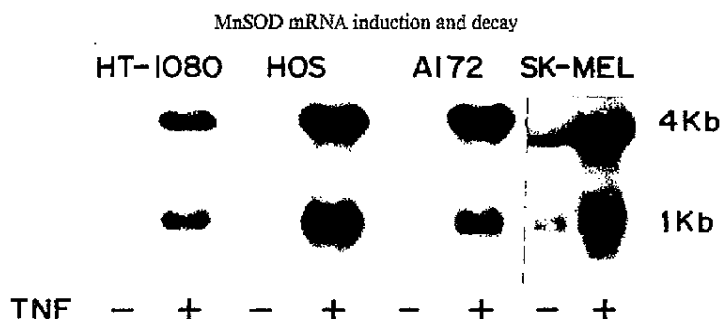
Fig. 1. Basal and TNF-induced levels of MnSOD mRNA in human cell lines. Confluent cultures of fibrosarcoma (HT-1080), osteosarcoma (HOS), glioblastoma (A172), and melanoma (SK-MEL) cells were treated with 10 ng/ml TNF for 4 h before RNA extraction. The MnSOD mRNAs were detected by Northern blot hybridization.

4-kb mRNA was more abundant than the 1-kb mRNA in A172 and HT-1080 cells). A similar tissue-specific variation in the relative abundance of the five rat MnSOD transcripts has previously been reported.[27] This variability in the relative proportion of the MnSOD mRNAs may be explained by different kinetics of induction and/or turnover.

To distinguish between these explanations, we examined the time course of induction of MnSOD mRNAs in A172 cells (Figs. 2A and B). The Northern blot in Fig. 2B was used to obtain the data in Fig. 2A after normalization against actin mRNA levels. Actin mRNA levels are not altered by TNF treatment.[12,13] Differences in actin mRNA levels in Fig. 2B were due to uneven loading of the RNA. In the first 2 h of TNF treatment, the 4-kb mRNA increased fivefold while

the 1-kb mRNA increased slightly (Fig. 2A). The level of the 4-kb transcript peaked after approximately 6 to 8 h when the 4- and 1-kb mRNA increased nine- and fourfold, respectively. Afterward, the level of the 4-kb mRNA progressively decreased, while that of the 1-kb mRNA increased slowly up to 12 h followed by a gradual decrease afterward (Fig. 2A).

The SOD activity gels showed that the induction of MnSOD mRNA by TNF (Fig. 2B) was closely followed by an increase in MnSOD enzymatic activity, while the CuZnSOD activity remained constant (Fig. 2C). Previous studies have suggested that increased MnSOD activity or immunoreactive protein are associated with elevated 1-kb mRNA levels.[5,20] It is not clear whether the increase in MnSOD activity between 2 and 4 h (Fig. 2C) is associated with the eight-
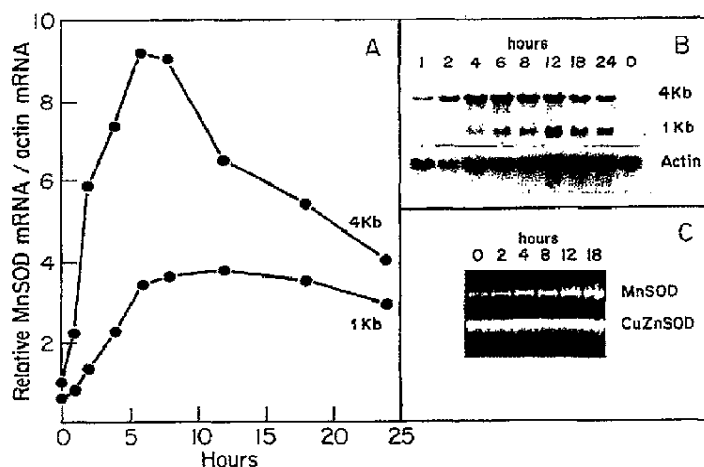

Fig. 2. Time course of MnSOD mRNA induction by TNF in A172 glioblastoma cells. Confluent cells were treated with 10 ng/ml TNF for the indicated times before RNA or protein extraction. (A) The levels of 4- and 1-kb MnSOD mRNAs were measured by a Betascope model 603 blot analyzer and normalized to actin mRNA levels. (B) Northern blot of MnSOD and actin mRNA used to obtain data in panel A. (C) Time course of MnSOD activity induction following TNF treatment for the indicated times; 25 µg of protein were loaded in each lane.
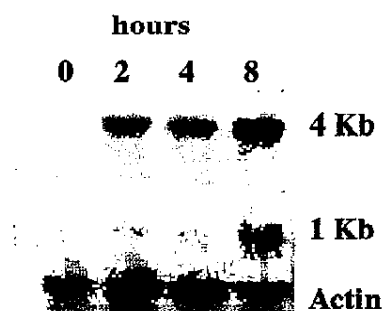
hours

0   2   4   8

Fig. 3. Time course of MnSOD mRNA induction by TNF in RIG human foreskin fibroblasts. Confluent cells were treated with 10 ng/ml TNF for the indicated times before RNA extraction. Northern blots of MnSOD and actin mRNAs are shown.

fold increase in the level of 4-kb mRNA or with the twofold increase in the level of the 1-kb mRNA species (Fig. 2C). However, since most of the MnSOD mRNA in A172 cells is of the 4-kb moiety up to 4 h, it seems likely that this mRNA is translated into functional MnSOD.

Transcription of the 4-kb mRNA appeared to precede that of the 1-kb mRNA. This was clearly shown by the absence of 1-kb mRNA after 1 h of TNF treatment in the Northern blot analysis of cytoplasmic RNA of A172 cells (Fig. 2B). This pattern of differential MnSOD mRNA induction was also demonstrated in human diploid fibroblast RIG cells (Fig. 3). Control cells not treated with TNF showed very little 1-kb MnSOD mRNA only after prolonged exposure of the autoradiograph (data not shown). In cytoplasmic RNA prepared after 1 and 2 h of TNF treatment, the 4-kb mRNA was already present, while little if any 1-kb mRNA was detectable (Fig. 3). These observations suggest that the 4-kb mRNA is preferentially produced in the early response to TNF.

We next examined whether the transient accumulation of the 4-kb mRNA in A172 cells was due to a turnover faster than that of the 1-kb mRNA. These cells were treated for 4 h with TNF, washed, and incubated in fresh medium (Fig. 4). The 4-kb mRNA rapidly decayed with an apparent half-life of ~4 h while the 1-kb mRNA remained relatively stable throughout this incubation. To establish that the fast turnover of the 4-kb mRNA was a general phenomenon, a similar experiment was performed with HOS cells. The level of both transcripts was about equal after 4 h TNF treatment in these cells; after incubation in TNF-free medium, there was a rapid decrease in the level of 4 kb mRNA with respect to 1 kb mRNA (Fig. 5). The apparent half-life of the 4-kb mRNA was ~2

h, while that of the 1-kb mRNA was ~10 h. These studies indicated that the 4-kb mRNA was degraded faster in HOS cells than in A172 cells (2 h vs. 4 h apparent half-life), while the 1-kb mRNA was equally stable in both cells. These different kinetics of degradation may account for the observation that the 4-kb mRNA is much more abundant in A172 cells than in HOS cells, relative to the 1-kb mRNA (Fig. 1).

To further characterize the differential degradation of the 4- and 1-kb MnSOD mRNA, we examined the effect of a transcriptional inhibitor, actinomycin D, on their decay. Treatment of TNF-stimulated HOS cells with actinomycin D inhibited degradation of both MnSOD mRNAs even after prolonged incubation (data not shown). Shull et al.[28] have recently demonstrated that actinomycin D increases the basal MnSOD mRNA levels 4.5-fold in hamster tracheal epithelial cells and suggested that turnover of MnSOD mRNA requires ongoing mRNA synthesis. Alternatively, actinomycin D might inhibit nucleases that target MnSOD mRNAs. Actinomycin D has also been shown to inhibit turnover of the short-lived *c-fos* mRNA by Shyu et al.,[29] who suggest that actinomycin D may selectively inhibit this turnover by intercalating within the AU-rich instability sequence found in the *c-fos* 3'UTR. Actinomycin D may similarly bind to the AU-rich sequence found in the 3'UTR of the 4-kb MnSOD mRNA.

Turnover of MnSOD mRNAs in a cell-free system

The above experiments clearly demonstrated the differential stability of the two MnSOD mRNAs in intact cells. We have also utilized a reticulocyte lysate to assess the stability of the MnSOD mRNAs in a cell-free system and to avoid the use of transcriptional inhibitors. The reticulocyte lysate has been previously utilized to demonstrate the instability of AU-rich mRNAs.[30,31] Poly(A+) RNA was isolated from total RNA of HOS cells treated for 4 h with TNF. This RNA showed the same pattern of 4- and 1-kb SOD mRNA observed in the experiments described above with cytoplasmic RNA. The poly(A+) RNA was incubated with unsupplemented reticulocyte lysate and the relative amounts of the two MnSOD mRNAs were measured by Northern blot analysis (Fig. 6). This experiment showed that 45% and 70% of the 4-kb mRNA disappeared after 15 and 30 min of incubation, respectively, while the level of the 1-kb mRNA and of GAPDH mRNA used as control did not change appreciably. No loss of the 4-kb mRNA occurred in an incubation without added reticulocyte lysate (data not shown).
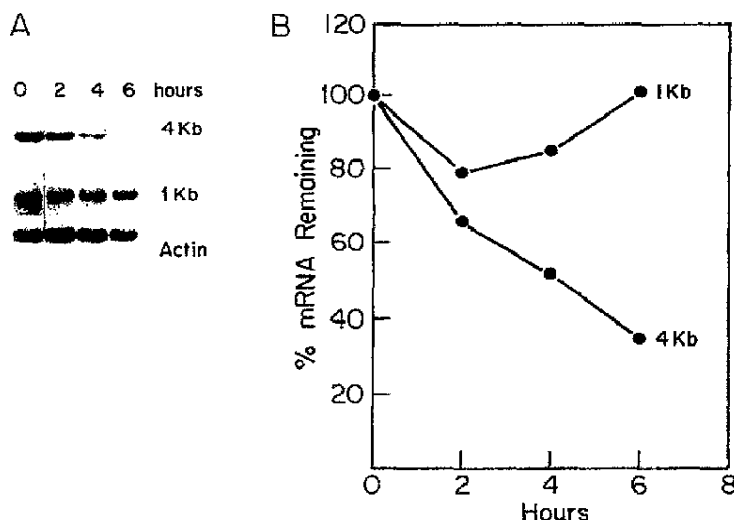
Fig. 4. Decay of 4- and 1-kb MnSOD mRNAs in A172 cells after TNF removal. (A) A172 cells were grown to confluence and treated for 4 h with 10 ng/ml TNF (time 0). The cells were then washed twice with fresh medium and further cultured in this medium. MnSOD and actin mRNAs were analyzed as described in Fig. 3. (B) Measurement of the 4- and 1-kb MnSOD mRNAs normalized to actin mRNA levels (see Fig. 1 for details). The MnSOD mRNA level at time 0 is taken as 100%.

DISCUSSION

The human MnSOD gene is transcribed into two distinct mRNA species of 4- and 1-kb that differ only in their 3'UTR. Both mRNAs are transcribed from a single gene,[32] by the use of alternate polyadenylation signals.[17] Recent studies have demonstrated the existence of at least five rat MnSOD transcripts derived from a single gene as a result of alternate polyadenylation.[27] We suggest here that the relative level of 4- and 1-kb MnSOD mRNA in different cells is determined by different kinetics of both synthesis and turnover. The 4-kb mRNA is expressed at a faster rate than the 1-kb mRNA, as shown by the rapid accumulation of high levels of 4-kb mRNA after TNF addition (Fig. 2A). The present study demonstrates, however, that the 4-kb mRNA has a much shorter half-life than the 1-kb mRNA in both intact cells and a cell-free system. This finding implies that differential mRNA stability is an important component in the regulation of MnSOD levels.

We would like to suggest a possible explanation for this regulatory pattern of MnSOD expression. A basal level of MnSOD activity is present in all the cells examined. Treatment with TNF or interleukin-1 induces a rapid increase in the level of MnSOD activity.[10,12,26,33] In our experiments, this increase in MnSOD activity occurred as early as 2 h after addition of TNF and closely followed the increase in the level of 4-kb mRNA (Fig. 2C). Therefore, the induction of the 4-kb mRNA coincides with a rapid increase in MnSOD activity. This enzymatic activity may have a protective role for cells that are exposed to free radicals produced by macrophages and granulocytes at sites of inflammation where TNF and interleukin-1 are secreted.[34] However, the short half-life of the 4-kb mRNA prevents the overexpression of MnSOD in cells continuously treated with TNF. It seems possible that such an overexpression of MnSOD may be harmful to cells. Previous studies have demonstrated that a high level of CuZnSOD or MnSOD may cause an imbalance in the cellular oxidant status that becomes detrimental to the cell or organism.[35,36] In any case, the relatively slow induction but long half-life of the 1-kb mRNA allows a gradual increase in MnSOD activity. Therefore, the level of MnSOD activity may be optimally regulated by producing at high rate an unstable mRNA and at low rate a stable mRNA coding for the same enzyme. The decreasing ratio of 4-kb to 1-kb mRNA after TNF treatment may reflect changing patterns of either alternate polyadenylation or mRNA stabilization. TNF is known to promote a transient stabilization of the short-lived glucose transporter and interleukin-1 mRNAs that contain AU-rich instability sequences in their 3'UTR.[37,38] This mRNA stabilization may account in part for the rapid accumulation of 4-kb MnSOD mRNA early after TNF treatment.
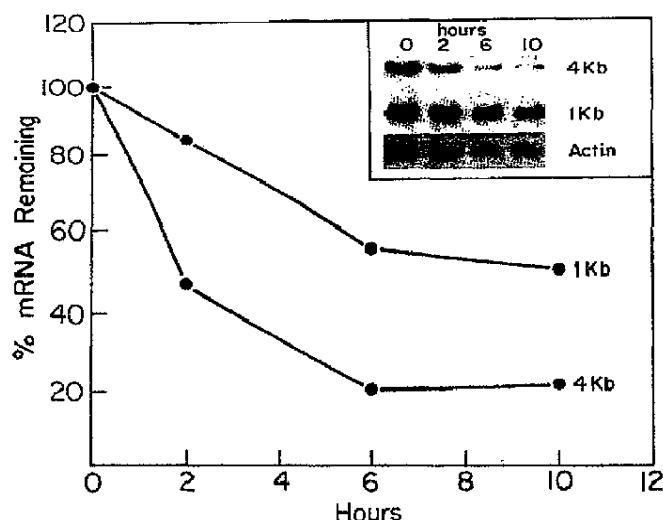
Fig. 5. Decay of 4- and 1-kb MnSOD mRNA in HOS cells after TNF removal. The cells were treated with TNF as described in Fig. 4. MnSOD mRNA levels were measured as described in Fig. 4. Northern blots of MnSOD and actin mRNAs are shown in the inset.

Previous studies have reported that other mRNAs containing identical coding regions but differing in the 3'UTR show different stability (e.g., insulin growth factor-1 [IGF-1] mRNAs of 7 and 1 kb differ in their turnover rate). The in vivo apparent half-life of the 7-kb mRNA is 4 h compared to the 14-h apparent half-life of the 1-kb mRNA.[31] Similarly, plasminogen activator inhibitor-1 (PAI-1) mRNAs of 3.4 and 2.2 kb have apparent half-lives of 0.85 and 2.5 h, respectively.[39] Recent studies have shown that insulin can stabilize the larger PAI-1 mRNA, while IGF-1 can increase the half-life of both PAI-1 mRNAs.[40] A common feature of the MnSOD, IGF-1, and PAI-1 mRNA is the presence of a long 3'UTR in the larger and less stable transcripts. Sequence comparison shows no significant homology among these 3'UTRs, except for a 60-base region of identity between the 4-kb MnSOD and the 3.4-kb PAI-1 mRNA corresponding to an *Alu* element.[41] It is unlikely that such *Alu* elements have any regulatory function because of their apparently random distribution throughout the human genome. However, AUUUA motifs are present in the 3'UTR of the large mRNA species, but not in the 3'UTR of the corresponding short mRNAs. The instability of the large mRNA species may be determined by these AUUUA motifs that are apparently involved in regulating the instability of mRNAs coding for lymphokines, cytokines, and proto-oncogenes.[19] Additional evidence for the involvement of

AU-rich regions in regulating mRNA stability was previously obtained by experiments in a cell-free system;[30] mRNA instability was correlated with the presence of AU-rich sequences in the 3'UTR. Our data in the reticulocyte cell-free system lend further support to this hypothesis. Therefore, AUUUA motifs in the 3'UTR of the 4-kb mRNA may be responsible for its instability.

Recent studies have suggested that specific protein factors modulate the turnover of mRNAs containing AUUUA motifs in the 3'UTR.[42–45] These factors may either stabilize or target mRNA for degradation. The binding activity of these factors was increased by treatments with phorbol esters,[43] TNF, c-AMP analogs, and a phosphatase inhibitor, okadaic acid;[45] their activity was abolished by the addition of oxidizing agents.[43] Clerch et al.[46] have also shown that a redox-sensitive mRNA binding protein is involved in regulating the stability of the mRNA for the antioxidant catalase. It remains to be established whether a specific mRNA binding factor modulates the stability of the MnSOD transcripts.

MnSOD is one of the key players in combatting superoxide-mediated damage in the mitochondria. The levels of this protein are relatively low under normal physiological conditions. However, during differentiation and in response to oxidants, cytokines, and lipopolysaccharides the MnSOD activity can increase 10–20-fold. An overabundance of MnSOD may re-
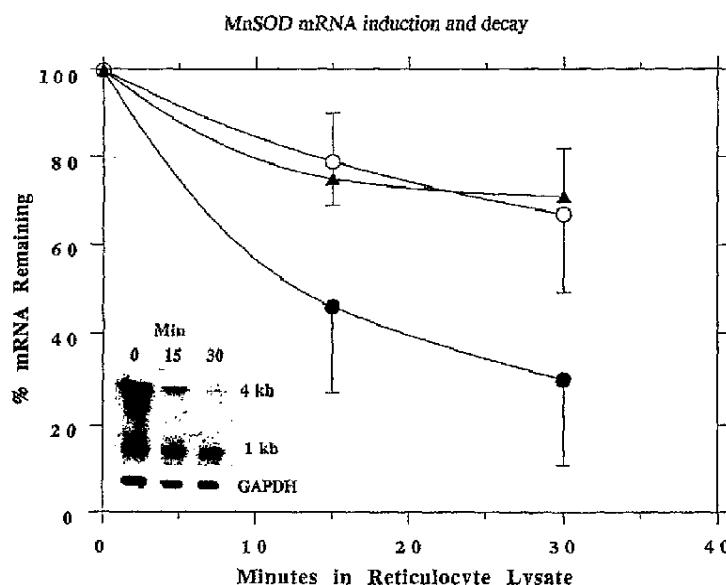
MnSOD mRNA induction and decay

Fig. 6. Decay of 4- and 1-kb MnSOD mRNA in a reticulocyte lysate cell-free extract. Poly (A+) RNA was incubated for the indicated times in a cell-free extract. Samples were taken at the indicated times and mRNA extracted as described in Materials and Methods. 4 kb (●), 1 kb (○), and GAPDH (▲) mRNA levels were measured by a Betascope blot-analyzer and represented as a percentage of time 0. Data correspond to the mean ± SEM of three different experiments. The inset shows one representative experiment.

sult in the accumulation of toxic levels of hydrogen peroxide and subsequent production of the highly reactive hydroxyl radical. MnSOD levels may be tightly regulated post-transcriptionally by adjusting the levels of stable and unstable mRNAs which code for this protein. Cells may respond to rapid onslaught of superoxide by rapidly increasing the levels of the unstable 4-kb mRNA followed by a gradual accumulation of the stable 1-kb mRNA, thereby preventing the levels of MnSOD from becoming deleterious to the cell.

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